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ALTERATIONS IN NUCLEOTIDE POOLS INDUCED BY 3-DEAZAADENOSINE AND RELATED COMPOUNDS

ROLE OF ADENYLATE DEAMINASE*

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Abstract—3-Deazaadenine, 3-deazaadenosine, and the carbocyclic analog of 3-deazaadenosine produced similar effects on nucleotide pools of L1210 cells in culture: each caused an increase in IMP and a decrease in adenine nucleotides and had no effect on nucleotides of uracil and cytosine. Concentrations of 50–100 μM were required to produce these effects. Although 3-deazaadenosine and carbocyclic 3-deazaadenosine are known to be potent inhibitors of adenosylhomocysteine hydrolase, the effects on nucleotide pools apparently are not mediated via this inhibition because they are also produced by the base, 3-deazaadenine, and because the concentrations required are higher than those required to inhibit the hydrolase. Cells grown in the presence of 3-deazaadenine or 3-deazaadenosine contained phosphates of 3-deazaadenosine (the mono- and triphosphates were isolated); from cells grown in the presence of the carbocyclic analog of 3-deazaadenosine, the monophosphate was isolated, but evidence for the presence of the triphosphate was not obtained. A cell-free supernatant fraction from L1210 cells supplemented with ATP catalyzed the formation of monophosphates from 3-deazaadenosine or carbocyclic 3-deazaadenosine, and a cell-free supernatant fraction supplemented with 5-phosphoribosyl 1-pyrophosphate (PRPP) catalyzed the formation of 3-deaza-AMP from 3-deazaadenine. Adenosine kinase apparently was not solely responsible for the phosphorylation of the nucleosides because a cell line that lacked this enzyme converted 3-deazaadenosine to phosphates. No evidence was obtained that the effects on nucleotide pools resulted from a block of the IMP–AMP conversion, but the results could be rationalized as a consequence of increased AMP deaminase activity. This explanation is supported by two observations: (a) coformycin, an inhibitor of AMP deaminase, prevented the effects on nucleotide pools, and (b) 3-deazaadenine decreased the conversion of carbocyclic adenosine to carbocyclic ATP and increased its conversion to carbocyclic GTP. The latter conversion requires the action of AMP deaminase and the observed effects can be rationalized by a nucleoside analog-mediated increase in AMP deaminase activity. Because these effects on nucleotide pools are produced only by concentrations higher than those required to inhibit adenosylhomocysteine hydrolase, they may not contribute significantly to the biological effects of 3-deazaadenosine or carbocyclic 3-deazaadenosine. These results therefore are primarily of interest in that they (a) suggest that AMP-deaminase activity in intact cells may be stimulated by certain purine and nucleoside analogs and (b) also point to the possibility that other analogs might be designed that would be even more effective and specific in modulating the activity of this enzyme.

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‡ Abbreviations: 3-deaza-Ade, 3-deazaadenine; 3-deaza-Ado, 3-deazaadenosine; *C*-Ado, 9-[β-DL-2α,3α-dihydroxy-4-β-(hydroxymethyl)cyclopentyl] adenine, the carbocyclic analog of adenosine; 3-deaza-*C*-Ado, 9-[β-DL-2α, 3α-dihydroxy-4-β-hydroxymethyl)cyclopentyl]-3-deaza-Ade, the carbocyclic analog of 3-deazaadenosine; 3-deaza-AraA, 9-β-D-arabinofuranosyl-3-deaza-Ade; *C*-ATP, the carbocyclic analog of ATP; *C*-GTP, the carbocyclic analog of GTP; MeMPR, 6-(methylmercapto)purine ribonucleoside; AMP-DA, adenosine-5′-phosphate deaminase (EC 3.5.4.6); HPLC, high pressure liquid chromatography; AdoHcy, *S*-adenosylhomocysteine; r.t., retention time; and PRPP, 5-phosphoribosyl 1-pyrophosphate.

3-Deazaadenosine (3-deaza-Ado)‡ and its carbocyclic analog (3-deaza-*C*-Ado) are potent inhibitors of adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1.) [1, 2]. A consequence of this inhibition is an accumulation of AdoHcy with a resulting inhibition of methylation reactions. The varied biological effects of 3-deaza-Ado have generally been attributed to inhibition of methylases [3], although at least one action of 3-deaza-Ado, namely inhibition of lymphocyte-mediated cytolysis, appears not to be mediated via inhibition of AdoHcy hydrolase [4].

In the course of examining 3-deaza-*C*-Ado for effects on nucleotide metabolism, we noted that, at concentrations higher than those required for inhibition of AdoHcy hydrolase, it produced a decrease in ATP and an accumulation of IMP. 3-Deaza-Ado produced the same effect. However, the effect apparently was not mediated via inhibition of AdoHcy hydrolase since 3-deazaadenine (3-deaza-Ade),

which does not inhibit this enzyme, had similar activity. We report here the details of these findings and of attempts to establish the biochemical basis for them, which have led to the conclusion that they can best be rationalized as a stimulation of the activity of AMP deaminase (AMP-DA).

MATERIALS AND METHODS

3-Deaza-AMP and 3-deaza-ATP. 3-Deaza-AMP was synthesized from 3-deaza-Ado by use of nucleoside phosphotransferase prepared from carrots as described by Brunngraber [5]. The product was assayed for purity by high pressure liquid chromatography (HPLC), and its dephosphorylation by 5′-nucleotidase showed that the phosphate was at the 5′-position. These procedures have been described elsewhere for the preparation of other nucleotides [6]. 3-Deaza-AMP was converted to 3-deaza-ATP by incubation with myokinase and nucleoside diphosphokinase; the procedure was essentially that used by Saunders *et al.* [7] for preparation of 3-deaza-GTP except that myokinase replaced GMP kinase in the reaction mixture. The product, 3-deaza-ATP, had a retention time of 32 min when subjected to HPLC on a Partisil 10-SAX column; it was obtained only in quantity sufficient to use as a chromatographic standard.

Other compounds and materials. 3-Deaza-Ade [8], 3-deaza-Ado [9], 3-deaza-*C*-Ado [2], 9-β-D-arabinofuranosyl-3-deazaadenine (3-deaza-AraA) [10] and the carbocyclic analog of adenosine (*C*-Ado, ± aristeromycin) [11] were synthesized in our laboratories. [8-^{14}C]Adenine (sp. act. 59 mCi/mmol) and [8-^{14}C]hypoxanthine (sp. act. 56 mCi/mmol) were obtained from Moravek Biochemicals, Inc., Brea, CA. Alkaline phosphatase from *Escherichia coli*, 5′-nucleotidase from *Crotalus adamanteus*, myokinase from rabbit muscle, and nucleoside diphosphokinase from baker's yeast were obtained from the Sigma Chemical Co., St. Louis, MO. Coformycin was a gift from Warner-Lambert/Parke-Davis Laboratories, Detroit, MI, and L-alanosine was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Experiments with cell cultures. The cell lines used were HEp-2 cells, L1210 mouse leukemia cells (designated L1210/0) and an L1210 subline (L1210/MeMPR) selected for resistance to 6-(methylmercapto)purine ribonucleoside and essentially devoid of adenosine kinase activity [12]. HEp-2 cells were grown in SRI-14 medium [13] and determinations of cytotoxicity were made by assay of effects on colony formation when cells were plated in the presence of various concentrations of inhibitors and the number of colonies formed were counted visually 10 days later [14]. The L1210 cell lines were maintained and grown in Fischer's medium [15], and determinations of cytotoxicities were the same as those described for HEp-2 cells except that the cloning was in soft agar. For metabolic experiments, L1210/0 and L1210/MeMPR cells were grown in suspension culture at a concentration of about 5×10^5 cells/ml. At the initiation of the experiments the cells were in exponential growth. Cells were harvested by centrifugation, washed free of medium with 0.85% NaCl solution, and then extracted with ice-cold 0.5 N $HClO_4$. To this ice-cold extract $KHCO_3$ was added and the precipitated $KClO_4$ was removed by centrifugation. The supernatant solution was lyophilized to dryness and the residue was taken up in H_2O; this solution was used for analysis by HPLC.

High pressure liquid chromatography. HPLC analyses were performed with either a Waters Associates (Milford, MA) model 202 apparatus or a Series 4 liquid chromatograph (Perkin–Elmer, Norwalk, CT). Analysis of nucleotides was accomplished with a Partisil-10 SAX anion exchange column (250 × 4.6 mm, Keystone Scientific, State College, PA); the gradient was either a linear or a slightly concave gradient (No. 7 on the Waters Associates programmer), from 5 mM $NH_4H_2PO_4$ (pH 2.8), to 750 mM $NH_4H_2PO_4$ (pH 3.7), and the flow rate was 2 ml/min. The linear gradient was used routinely; when the No. 7 gradient was used, its use is specifically noted in the legends to the figures. Nucleosides were analyzed on a reversed phase Spherisorb ODS 5 μM column (250 × 4.6 mm, Keystone Scientific, or Phase-Sep., Norwalk, CT) with an eluant consisting of 5 mM $NH_4H_2PO_4$ (pH 2.8) and acetonitrile (95:5, v/v) and at a flow rate of 1 ml/min. The ultraviolet absorption spectra of selected peaks were determined with a Perkin–Elmer model LC-75 stop-flow scanner or with a model 2140 rapid spectral detector (LKB Instruments, Gaithersburg, MD).

Conversion of 3-deaza-Ade, 3-deaza-Ado and 3-deaza-C-Ado to nucleotides in cell-free systems. Crude enzyme preparations, shown in other studies to contain activities of purine phosphoribosyltransferases and nucleoside kinases, were used to examine the conversion of 3-deaza-Ade, 3-deaza-Ado and 3-deaza-*C*-Ado to their nucleotides. L1210 cells grown in culture were harvested by centrifugation, washed free of medium, and homogenized in 3 vol. of 0.05 M Tris, pH 7.5 (motor-driven pestle). The homogenate was centrifuged at 100,000 *g* for 1 hr, and the supernatant fraction was dialyzed against three changes of 0.05 M (pH 7.5) Tris. For determination of the conversion of 3-deaza-Ade to 3-deaza-AMP, the reaction mixture contained 40 nmol of $Mg_2PRPP \cdot 2H_2O$, 40 nmol of $MgCl_2$ and 10 nmol of 3-deaza-Ade in a final volume of 0.100 ml. For determination of conversion of 3-deaza-Ado or 3-deaza-*C*-Ado to nucleotides, the reaction mixture contained 9 nmol ATP, 4.5 nmol $MgCl_2$, 90 nmol Tris (pH 8.4), 27 nmol NaF, 0.018 nmol deoxycoformycin, and 0.54 nmol of 3-deaza-Ado or 3 deaza-*C*-Ado in a final volume of 1.8 ml. At intervals of 5 min, portions of the reaction mixtures were removed, and the reaction was stopped by immersion in a boiling water bath. After removal of the protein by centrifugation, the supernatant fraction was analyzed by HPLC on an anion exchange column. This column separated 3-deaza-AMP [retention time (r.t.) 4.0 min] or 3-deaza-*C*-AMP (r.t. 4.0 min) from AMP (r.t. 3.5 min); the amounts of nucleotides formed were determined by integration of areas under the peaks.

Table 1. Cytotoxicities of 3-deaza-Ade, 3-deaza-Ado, 3-deaza-*C*-Ado and 3-deaza-AraA

	IC_{50} (μM)		
	HEp-2 Cells	L1210/0 Cells	L1210/MeMPR Cells
3-Deaza-Ade	9.7 (6.7–12.6)	50 (21–69)	70 (48–90)
3-Deaza-Ado	7.7 (6.8–8.6)	5.4 (2.6–7.1)	13 (9.4–18.8)
3-Deaza-*C*-Ado	1.1 (1.0–1.1)	0.6 (0.2–1.1)	0.8 (0.4–1.2)
3-Deaza-AraA	70 (64–75)	28 (16–38)	60 (58–62)

The IC_{50} values (concentrations giving 50% inhibition) were determined from the number of colonies formed after a 10-day exposure of cells to various concentrations of the inhibitor (see text). The values shown are the average of two or more independent experiments in which each concentration was assayed in triplicate. The numbers in parentheses show the range of values for all of the experiments performed.

RESULTS

Cytotoxicities. Although 3-deaza-Ado and 3-deaza-*C*-Ado have been studied in many cell systems, it was desirable to determine cytotoxicities of these agents and 3-deazaadenine and 3-deaza-AraA in the same experiments. As shown in Table 1, 3-deaza-*C*-Ado was the most toxic of the four compounds to both L1210 cell lines and to HEp-2 cells. The toxicities of all four agents to L1210/0 cells and to L1210/MeMPR cells were of the same order; this result indicates that phosphorylation by adenosine kinase is not essential for the toxicity of the nucleoside analogs. There were no very large differences in sensitivities of HEp-2 and L1210 cells to the nucleoside analogs, but HEp-2 cells were significantly more sensitive than L1210 cells to 3-deaza-Ade.

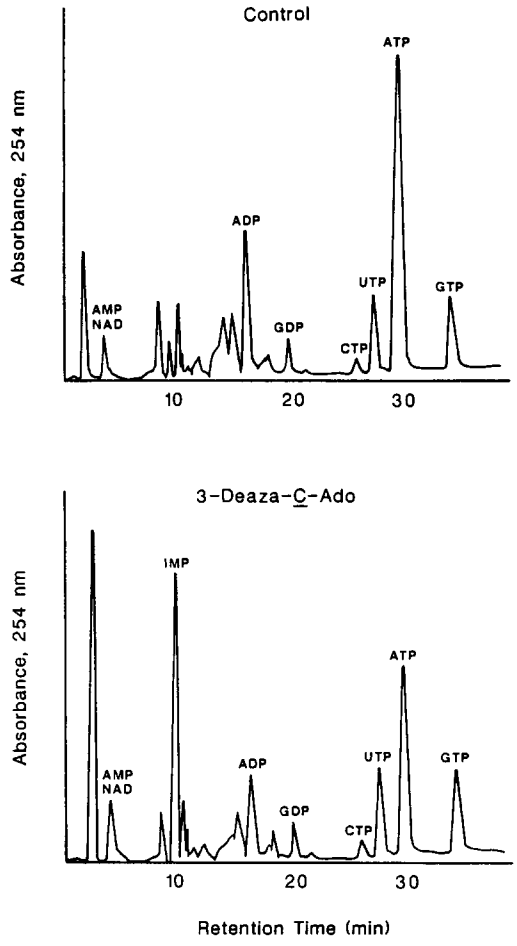

Fig. 1. Effects of 3-deaza-*C*-Ado on the nucleotide pools of L1210/0 cells. To L1210/0 cells in culture (approximately 5×10^5 cells/ml), 3-deaza-*C*-Ado was added to yield a final concentration of 94 μM. Cells were harvested 4 hr thereafter, and the nucleotide content was analyzed by HPLC on an anion exchange column (see text).

Effects of 3-deaza-Ade, 3-deaza-Ado, and 3-Deaza-C-Ado on nucleotide pools. The first evidence of an effect of a 3-deaza-Ade derivative on nucleotide pools was noted in experiments with 3-deaza-*C*-Ado (Fig. 1). Treatment of L1210/0 cells with 3-deaza-*C*-Ado caused a decrease in ATP and ADP and an increase in a peak in the monophosphate area (r.t. approx. 10 min); other nucleotide pools changed little or not at all. The peak in the monophosphate area that was increased in 3-deaza-*C*-Ado-treated cells was suspected, because of its r.t., to be IMP. Scanning of this peak with a stop-flow scanner showed a symmetrical absorption curve with a λ_{max} at 247 nm. This spectrum was not distinguishable from that of IMP determined in the same buffer. Further evidence that this peak was IMP was obtained in two types of experiments. In the first of these, the cell extract was treated with alkaline phosphatase prior to chromatography on a reversed phase column. The resulting chromatogram showed that the extract from treated cells, but not that from control cells, contained a large peak with the r.t. and UV absorption spectrum of inosine. In the second type of experiment, IMP was added to the cell extract prior to chromatography; this addition specifically increased the size of the 10-min peak (results not shown). Following this observation with 3-deaza-*C*-Ado, similar experiments were performed with other 3-deazapurines with the results shown in Table 2. The chromatograms for the experiments of Table 2 are not shown, because those for 3-deaza-Ade and 3-deaza-Ado did not differ significantly from that for 3-deaza-*C*-Ado shown in Fig. 1. 3-Deaza-Ade and 3-deaza-Ado were similar to 3-deaza-*C*-Ado in producing a decrease in ATP and an increase in IMP;

Table 2. Effects of 3-deaza-Ade and some derivatives on nucleotide pools of L1210 cells

Compound added	Nucleotide pool size (% of control)				
	Hypoxanthine	Adenine	Guanine	Uracil	Cytosine
3-Deaza-Ade	806	56	107	109	92
3-Deaza-Ado	420	43	109	92	100
3-Deaza-C-Ado	770	59	107	115	111
3-Deaza-AraA	98	88	100	105	111

The concentration of each of the 3-deazapurines was 100 μM. The experiments were performed as described in the legend to Fig. 1; the cells were harvested 4 hr after addition of the inhibitors. The nucleotide pools consist of the following moieties: hypoxanthine nucleotides, IMP; adenine nucleotides, AMP + NAD + ADP + ATP; guanine nucleotides, GDP + GTP; uracil nucleotides, UTP; cytosine nucleotides, CTP. Each experiment had its own control. The sizes (nmol/10^9 cells) of pools of control cells were: adenine nucleotides, 2201–2470; guanine nucleotides, 389–617; CTP, 213–352; UTP, 366–374; and IMP, 47–330.

3-deaza-araA was without significant effect on nucleotide pools. The pools of IMP were usually low in control cells and their quantitation consequently was subject to some error, which also is inherent when small pool sizes are expressed as percent of control. Because of this fact, the apparently smaller effect of 3-deaza-Ado on IMP buildup, as compared to those produced by 3-deaza-Ade and 3-deaza-C-Ado, probably is not significant and the results should be taken only to indicate that a considerable accumulation of IMP was seen with all three inhibitors. In these and in later experiments these inhibitors consistently produced an increase in IMP regardless of the size of this pool in control cells.

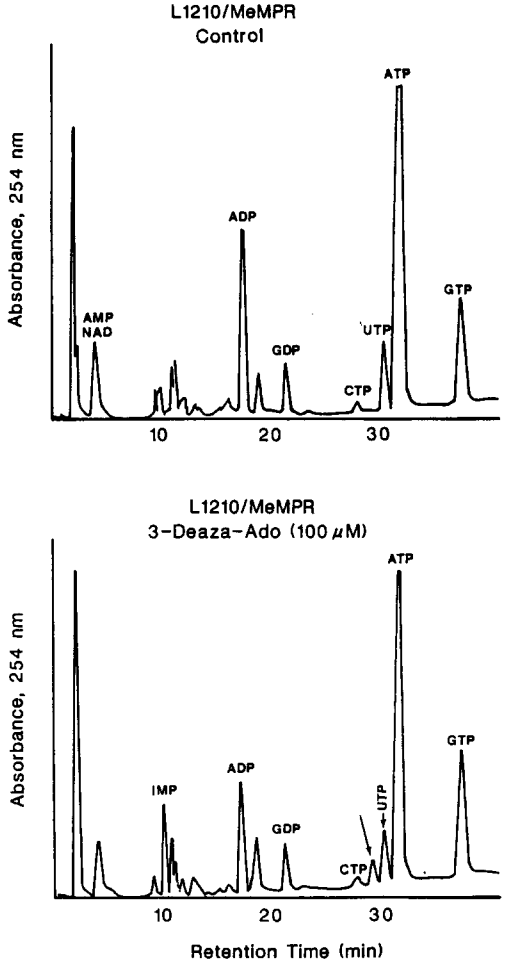

Fig. 2. Effects of 3-deaza-Ado on nucleotide pools of L1210/MeMPR cells. The experiments were performed as described in the legend to Fig. 1, except that L1210/MeMPR cells were used. The arrow marks a new peak appearing in the triphosphate area which is 3-deaza-ATP (see Fig. 5 and text).

Effects of 3-deazapurine derivatives in cells deficient in adenosine kinase. To determine if adenosine kinase-mediated phosphorylation was involved in the effects of the 3-deazapurines on nucleotide pools, studies similar to those shown in Fig. 1 were performed with L1210/MeMPR cells. Figure 2 shows the effects of 3-deaza-Ado in L1210/MeMPR cells, which were similar to those observed in cells of the parent line: ATP decreased and IMP increased. However, the increase in IMP was not as great as that observed in L1210/0 cells (Fig. 1). 3-Deaza-Ade and 3-deaza-C-Ado gave similar results (not shown). In Fig. 2 note is made of the presence of a new peak (marked with an arrow) eluting between CTP and UTP; the characterization of this peak is described later.

Effects of time of exposure and concentrations of 3-deaza-Ade or 3-deaza-Ado on nucleotide pools. Experiments were performed with 3-deaza-Ade and 3-deaza-Ado to determine the concentrations required to produce effects on nucleotide pools (Table 3, part A). Concentrations of 10 and 25 μM of either agent failed to produce marked effects on pools of ATP or IMP. At a concentration of 50 μM, 3-deaza-Ade produced a 50% decrease in the ATP pool and a 2-fold increase in the IMP pool; the magnitudes of these effects were not changed when the concentration was raised to 100 μM. 3-Deaza-Ado was somewhat less effective than 3-deaza-Ade; a concentration of 100 μM was required to produce a marked effect on ATP, although at a concentration of 50 μM it increased the IMP pool 2-fold.

3-Deaza-Ade was used in experiments to determine the time course of the changes in nucleotide pools (Table 3, part B). Significant effects on ATP were produced in 1 hr and maximum effects were

Table 3. Pools of ATP and IMP as a function of (A) concentration of 3-deaza-Ade or 3-deaza-Ado and (B) as a function of time of exposure to 3-deaza-Ade

		Nucleotide Pools (% of Control)	
		ATP	IMP
A.	Concn (μM)		
3-Deaza-Ade	10	112	76
	25	94	163
	50	51	189
	100	55	240
3-Deaza-Ado	10	96	110
	25	86	71
	50	82	205
	100	67	171
B.	Time		
3-Deaza-Ade (100 μM)	(hr)		
	1	78	364
	2	71	349
	4	59	326
	6	58	358
	12	69	346
	18	57	128

For both experiments A and B, L1210 cells were in logarithmic growth (approximately 5×10^5 cells/ml) at the initiation of the experiment. In A, cells from the same batch were subdivided into ten flasks, and 3-deaza-Ade or 3-deaza-Ado was added at the indicated concentrations and the other two flasks served as controls. After 2 hr the cells were harvested and extracted, and the extracts were subjected to HPLC on an anion exchange column. In B, cells from a single batch were subdivided equally into each of twelve flasks; to six of these 3-deaza-Ade was added at a concentration of 100 μM. At 1, 2, 4, 6, 12 and 18 hr thereafter cells from one treated and one control flask were harvested and assayed for nucleotide pools by HPLC. See text for details of the analyses. A single control served for experiment A; in experiment B, each time point had a separate control. The range of control values (nmol/10^9 cells) were: adenine nucleotides, 2043–3025; guanine nucleotides, 579–764; CTP, 188–605; UTP, 367–818; IMP, 83–378; the lower limits of these ranges were for the 18 hr experiment. See Table 2 for constituents of nucleotide pools.

noted after 4 hr. Maximum effects on IMP were observed at 1 hr; effects on both pools were still evident at 12 hr. At 18 hr the pool of ATP was still depressed, whereas the pool of IMP had fallen almost to control levels.

Effects on utilization of hypoxanthine and adenine. 3-Deaza-Ade decreased incorporation of [^{14}C]Hyp into ATP and produced an accumulation of [^{14}C]IMP without affecting its conversion to GTP (Fig. 3, panels A and B). Similar experiments were performed with 3-deaza-Ado and 3-deaza-*C*-Ado; the results were similar to those found with 3-deaza-Ade and are therefore not shown. 3-Deaza-Ado was used in a similar study with [^{14}C]Ade (Fig. 3, panels C and D). With this precursor there was also an increase in ^{14}C in IMP and a decrease of ^{14}C incorporation into ATP; the labeling of GTP was very low in the control and was somewhat increased in the treated cells. The differences in retention times between the metabolites in the hypoxanthine experiments and the adenine experiments are due to the fact that different columns were used.

Experiments with alanosine (Fig. 4). Alanosine is a known inhibitor of the conversion of IMP to adenylosuccinate [16–18]. It was used in this study to provide data on the effects of a block at this site on the utilization of labeled adenine in the expectation that a comparison of its effects with those of the 3-deazapurine derivatives might indicate whether a block at this site was consistent with the observed effects of the 3-deazapurines. Prior to this study an experiment was performed with [^{14}C]Hyp to make certain that alanosine inhibited the synthesis of adenine nucleotides in L1210 cells. As shown in Fig. 4 (panels A and B), alanosine sharply decreased the incorporation of [^{14}C]Hyp into ATP and ADP and increased its incorporation into GTP and GDP; this is the result to be expected of an agent that blocks the IMP–AMP conversion. In contrast, alanosine had no effects on the conversion of [^{14}C]Ade to adenine nucleotides (Fig. 4, panels C and D). The lack of effect of alanosine on the metabolism of adenine obviously differs from the results with 3-deaza-Ado (Fig. 3). The retention times of peaks in panels A and B are somewhat shorter than those of the same peaks in panels C and D; this is because different gradients were used for the two experiments (see legend to Fig. 4).

Formation of analog phosphates in intact cells. The formation of what appeared to be a new triphosphate

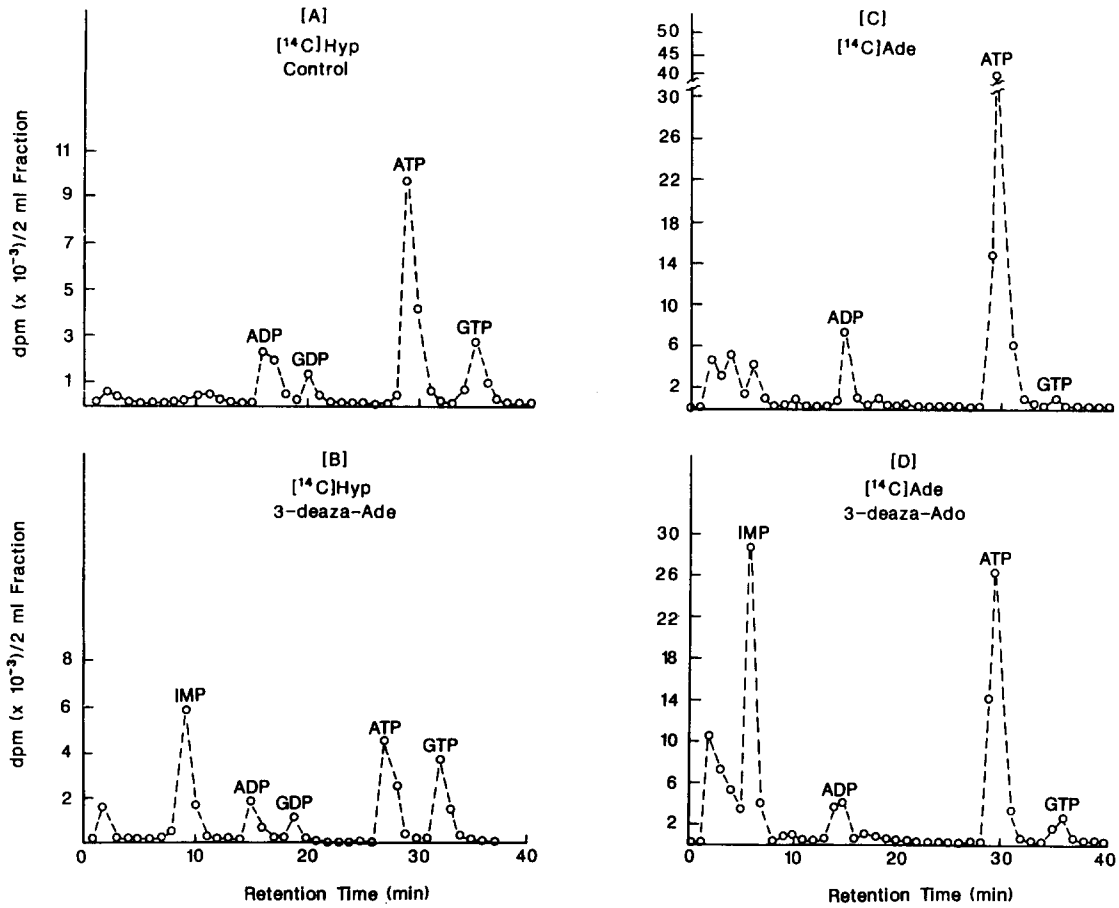

Fig. 3. Effects of 3-deaza-Ade or 3-deaza-Ado on the conversion of [^{14}C]hypoxanthine or [^{14}C]adenine to nucleotides. To cultures of L1210/0 cells (approx. 5×10^5 cells/ml), 3-deaza-Ade or 3-deaza-Ado was added to a final concentration of 100 μM, followed 0.5 hr later with [8-^{14}C]hypoxanthine or [8-^{14}C]adenine at a concentration of 0.025 μCi/ml; controls received only the labeled precursors. One hour after addition of [^{14}C]hypoxanthine and 2 hr after addition of [^{14}C]Ade the cells were harvested and extracted, and the extracts were subjected to HPLC on an anion exchange column as described in the text; different columns were used for the experiments with hypoxanthine and for those with adenine. Fractions of 2 ml were collected and assayed in a liquid scintillation spectrometer. The UV absorption spectra (not shown) and retention times were used to assign identities to the radioactive peaks.

of 3-deaza-Ado in the experiment of Fig. 2 prompted a reexamination of the metabolism of 3-deaza-Ado, because this product had not been observed in experiments carried out up to this time. In a series of experiments with 3-deaza-Ade and 3-deaza-Ado in L1210/0 cells, we found that this peak was consistently present, but was not always resolved: in some experiments it co-eluted with CTP and in others it appeared as a shoulder on the descending part of the CTP peak. The variability in the resolution of this peak probably results from the decrease in efficiency of a column during use, which is a well known phenomenon. We were able to resolve this peak consistently, even on "old" columns, by changing the pH of buffer B from 3.7 to 3.5; under these conditions UTP co-eluted with ATP and 3-deazaATP eluted between CTP and UTP-ATP. Figure 5 shows chromatograms of extracts of L1210/0 cells that were grown in the presence of 3-deaza-Ade (panel B), 3-deaza-Ado (panel C), or 3-deaza-*C*-Ado (panel D). These extracts were chromatographed under standard conditions (pH of buffer B = 3.7) on a "new" column. New peaks, indicated by arrows, appeared in the monophosphate and triphosphate areas in the chromatograms for both the 3-deaza-Ade- and 3-deaza-Ado-treated cells. These peaks had the same retention times as the known samples of 3-deaza-AMP and 3-deaza-ATP, prepared as described in Materials and Methods. In the cells treated with 3-deaza-*C*-Ado (panel D), the AMP–NAD peak had a shoulder that was not present in controls, but there was no evidence for a new peak or shoulder in the triphosphate area. Each of these new peaks or shoulders from all three precursors were scanned and gave UV absorption spectra characteristic of 3-deaza-Ado, i.e. an absorption maximum at 262 nm and an unsymmetrical peak with a distortion to the right of the maximum. These new peaks or shoulders thus appear to be the mono- and triphosphates of 3-deaza-

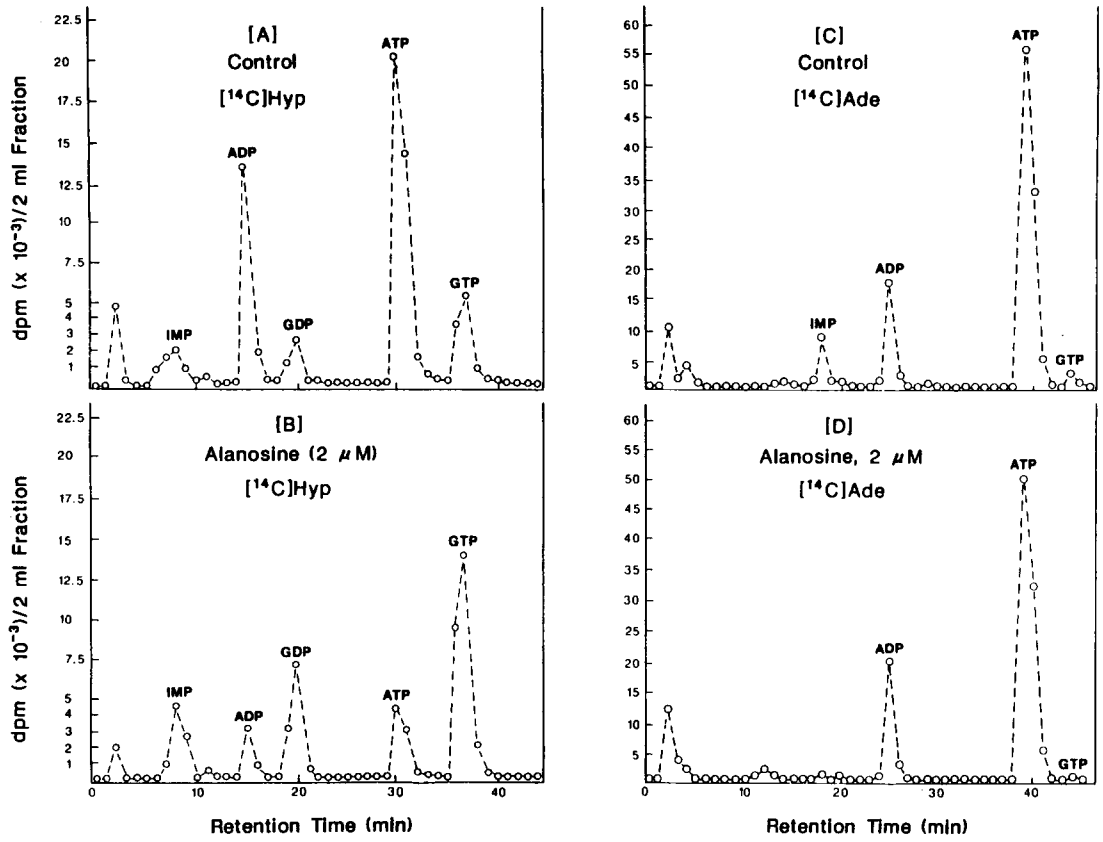

Fig. 4. Effects of alanosine on the conversion of [8-^{14}C]hypoxanthine and [8-^{14}C]adenine to nucleotides. The experiments were performed with L1210/0 cells as described in the legend of Fig. 3. Alanosine was added 0.5 hr before the addition of the labeled precursors; cells were harvested 1 hr after the addition of the labeled compounds. In the experiments of panels A and B, elution was achieved with a linear gradient; in those of panels C and D a concave gradient (No. 7) was used.

Ado (Fig. 5B and 5C) and the monophosphate of 3-deaza-*C*-Ado (Fig. 5D). To confirm the identities of these peaks, additional types of experiments were performed.

In the first of these, that portion of the monophosphate area (from both control and treated cells) eluting from 3 to 6 min was collected and lyophilized to dryness. The residue was taken up in H_2O and treated for 4 hr with alkaline phosphatase, after which the reaction was stopped by immersion of the reaction tube in a boiling H_2O bath for 2 min. The precipitate was removed by centrifugation and the supernatant solution was subjected to reversed phase chromatography. The chromatograms of the samples from treated cells showed the presence of a peak that was not present in the sample from control cells; these peaks had retention times and UV absorption spectra the same as that of 3-deaza-Ado or 3-deaza-*C*-Ado. Addition of the nucleosides to the sample prior to chromatography resulted in specific reinforcement of the peaks on the reversed phase chromatograms. In a second type of experiment, the entire cell extract was treated with alkaline phosphatase, and the resulting mixture of nucleosides was subjected to reversed phase chromatography as described above. The extract from each sample of treated cells showed only one peak that was not present in control cells, and this peak had the r.t. of 3-deaza-Ado or 3-deaza-*C*-Ado (not shown).

Although the monophosphate of 3-deaza-*C*-Ado was present, the ion exchange chromatogram (Fig. 5D) showed no evidence of a peak of 3-deaza-*C*-Ado-triphosphate. Scanning of the peaks of the natural triphosphates at multiple points on each peak gave no evidence of another substance co-eluting. In addition, no new peak was evident when the pH of the buffer was changed as described above. Nevertheless, the possibility remained that the triphosphate of 3-deaza-*C*-Ado might be present and undetected by these methods. Therefore an additional experiment was performed in which cells were grown for 1 hr in the presence of high concentrations (500 μM) of 3-deaza-*C*-Ado and the nucleotides were separated by anion exchange chromatography. The triphosphates (CTP through GTP) were collected and freed of buffer salts by adsorption on charcoal (Norit A) from which the nucleotides were eluted with ammoniacal-ethanol (ethanol:NH_4OH:H_2O (65:2:33, by vol.) [19]). The triphosphates were treated with alkaline phosphatase and the resulting nucleosides were chromatographed on a reversed phase column: no 3-deaza-*C*-Ado

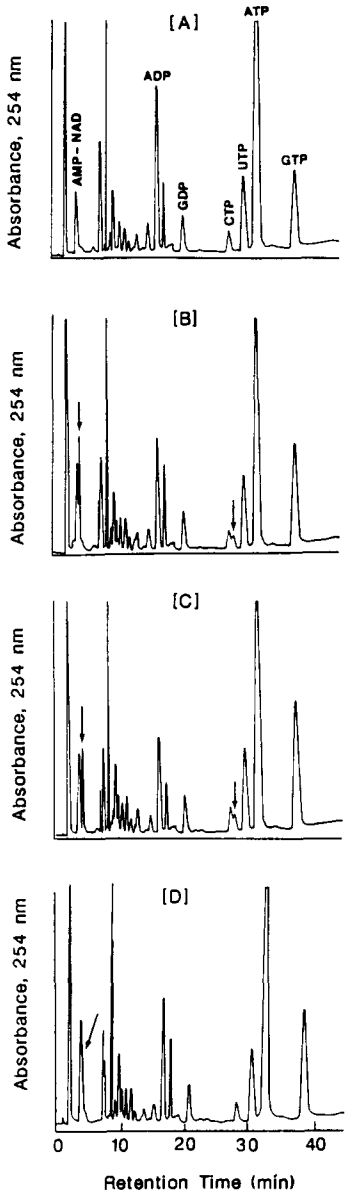

Fig. 5. Metabolism of 3-deaza-Ade, 3-deaza-Ado and 3-deaza-*C*-Ado by L1210 cells. L1210/0 cells were grown in the presence of the 3-deazapurine derivative (100 μM) for 4 hr, and extracts were prepared as described in the text. The extracts were subjected to chromatography on an anion exchange column. (A) Control cells, grown concurrently; (B) 3-deaza-Ade-treated cells; (C) 3-deaza-Ado-treated cells; and (D) 3-deaza-*C*-Ado-treated cells. The arrows mark new peaks or shoulders appearing in the treated cells.

could be detected. These experiments do not demonstrate unequivocally the absence of 3-deaza-*C*-Ado triphosphate, but they do indicate that, if present, it is in a very small amount.

Conversion of 3-deaza-Ade, 3-deaza-Ado, and 3-deaza-C-Ado to nucleotides in cell-free extracts. Since the 3-deazapurine derivatives were converted to nucleotides in intact cells, it was probable that 3-deaza-Ade was a substrate for a phosphoribosyltransferase and that 3-deaza-Ado and 3-deaza-*C*-Ado were substrates for a kinase or a phosphotransferase. To confirm the existence of pathways for conversion of these compounds to nucleotides, experiments were performed with cell-free extracts from L1210/0 cells. In the experiment with 3-deaza-Ade, the extract was supplemented with PRPP and Mg^{2+}, and in the experiments with 3-deaza-Ado and 3-deaza-*C*-Ado, with ATP and Mg^{2+}; nucleotide formation was monitored by HPLC. Nucleotides were formed from all three substrates, and the rates of formation were linear for 40 min. The rate of nucleotide formation was 100–150 nmol/mg protein/hr for 3-deaza-Ado and 3-deaza-*C*-Ado and 15 nmol/mg protein/hr for 3-deaza Ade (results not shown).

Effect of coformycin on 3-deaza-Ado-induced alteration of nucleotide pools. A possible explanation of the observed effects of the 3-deaza-Ade derivatives on nucleotide pools is a stimulation of AMP deaminase (AMP-DA) activity. To determine if AMP-DA was involved, we performed experiments with coformycin. We have shown in earlier studies that in intact L1210 cells coformycin at a concentration of 35 μM inhibits almost completely the conversion of certain nucleoside analogs (for example *C*-Ado [12] and 8-amino-6-fluoropurine ribonucleoside [20]) to the corresponding analogs of inosine and guanosine phosphates; this effect probably results from an inhibition by coformycin of the action of AMP-DA on the monophosphates of these nucleosides. (Adenosine deaminase is also inhibited at this concentration of coformycin.) Figure 6 shows the results of an experiment designed to determine the influence of coformycin on the effects of 3-deaza-Ade on nucleotide peaks. Figure 6C shows the effects of 3-deaza-Ado alone, namely the expected decrease in ATP and the increase in IMP as compared to controls (Fig. 6A). Figure 6B shows that coformycin alone had no specific effect on any nucleotide pool. As shown in Fig. 6D, the presence of coformycin completely prevented the effects of 3-deaza-Ado: the ATP was restored to normal levels and the IMP essentially disappeared.

Effects of 3-deaza-Ade on the metabolism of C-Ado. Figure 7A shows the metabolism of *C*-Ado in L1210/0 cells. As we have shown earlier [12] this agent is metabolized to phosphates of *C*-Ado and of carbocyclic guanosine. The presence of 3-deaza-Ade significantly altered the metabolism of *C*-Ado: the amount of *C*-ATP formed was decreased sharply and the conversion to the carbocyclic analog of GTP (*C*-GTP) was increased markedly (Fig. 7B).

DISCUSSION

The fact that 3-deaza-Ade, 3-deaza-Ado and 3-deaza-*C*-Ado produced the same effects on nucleotide pools implies a common mechanism of action. When these effects were first noted, it was thought that 3-deaza-Ado was not phosphorylated [21], and we considered it most likely that these agents as such (i.e. without conversion to phosphates) blocked the IMP–AMP conversion. Such a block obviously would account for a decrease in ATP and an accumulation of IMP. However, no inhibition of adenylosuccinate synthase or adenylosuccinate lyase could

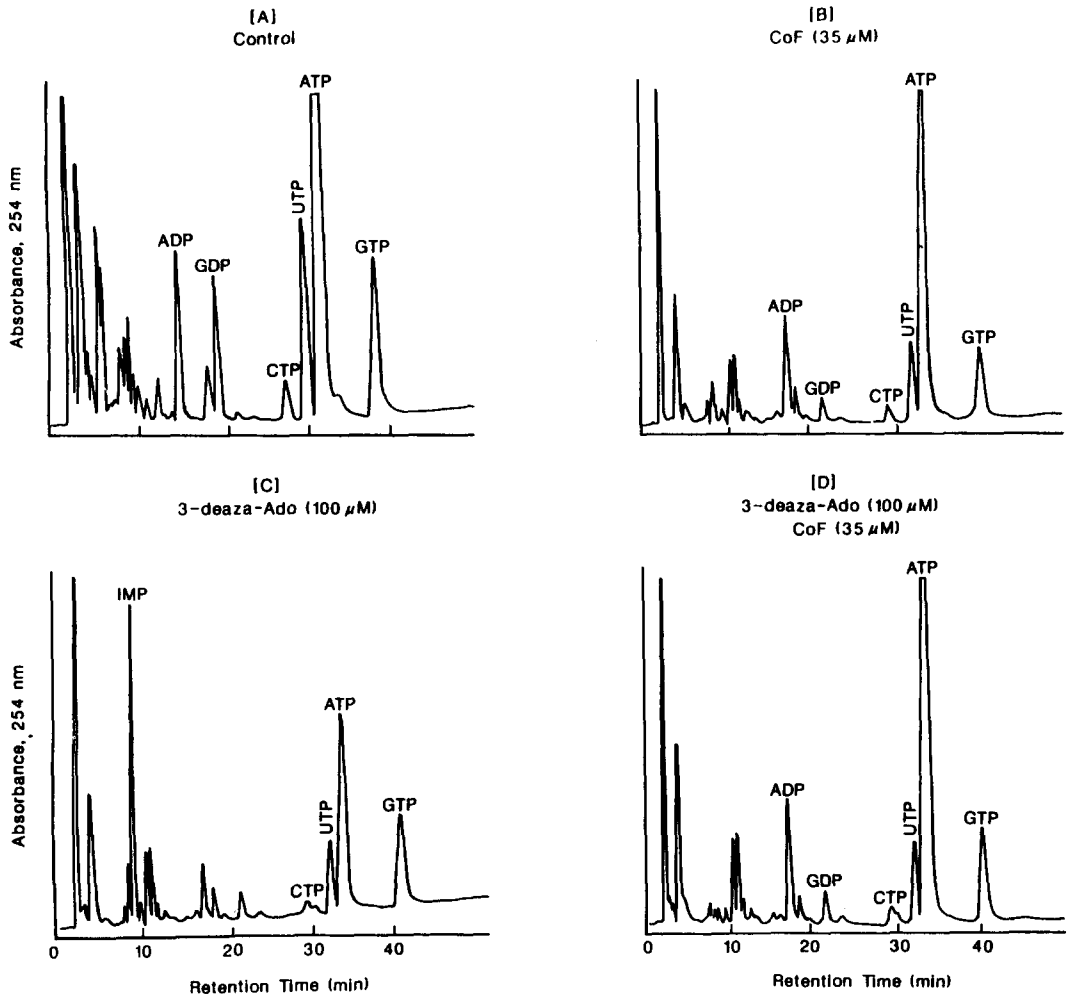

Fig. 6. Reversal by coformycin of 3-deaza-Ado-induced effects on nucleotide pools. L1210/0 cells in culture (approximately 5×10^5 cells/ml) were treated with: (A) nothing (controls); (B) coformycin; (C) 3-deaza-Ado; (D) coformycin plus 3-deaza-Ado. Coformycin was added 1 hr before the addition of 3-deaza-Ado, and cells were harvested 2 hr after addition of 3-deaza-Ado. Extracts of the cells were made and subjected to HPLC on an anion exchange column as described in the text. The distortion of the CTP peaks in panels C and D was due to the presence of the triphosphate of 3-deaza-Ado (see Fig. 5).

be shown with 3-deaza-Ade, 3-deaza-Ado, or 3-deaza-*C*-Ado.* The subsequent finding that nucleotides were formed from 3-deaza-Ade and 3-deaza-Ado suggested that the observed effects might be mediated by a nucleotide, and the observation that the effects of the 3-deazapurine derivatives could be prevented by coformycin provided evidence that the action of AMP-DA was required for the observed effects. Although this activity of coformycin in preventing effects on nucleotide pools is not rigorous proof that these effects result from inhibition of AMP-DA, the evidence is strong that this is so. This evidence consists of the facts that (a) coformycin is itself an inhibitor of AMP-DA [22] and in intact cells may be converted to the phosphate [23] which is an even more potent inhibitor [24, 25], and (b) in L1210 cells we have shown that coformycin (at the same concentration used in the present study) modifies the metabolism of nucleosides of certain 6-substituted purines in a way that can best be interpreted as resulting from inhibition of AMP-DA [12, 20].

There are two obvious mechanisms that would require the participation of AMP-DA in producing the observed alterations of nucleotide pools: (a) the active inhibitors might be 3-deaza-IMP or the carbocyclic analog of 3-deaza-IMP formed by the action of AMP-DA on 3-deaza-AMP or 3-deaza-*C*-AMP, or (b) the activity of AMP-DA might be increased in the presence of nucleotides of 3-deaza-Ade. Unfortunately, 3-deaza-IMP and the carbocyclic analog of 3-deaza-IMP were not available for a direct test of the first of these possibilities. However, the effects of 3-deaza-Ade and its derivatives on the utilization of labeled adenine are inconsistent with a block of the IMP–AMP conversion. Thus, 3-deaza-

* E. L. White and L. L. Bennett, Jr., unpublished observations.

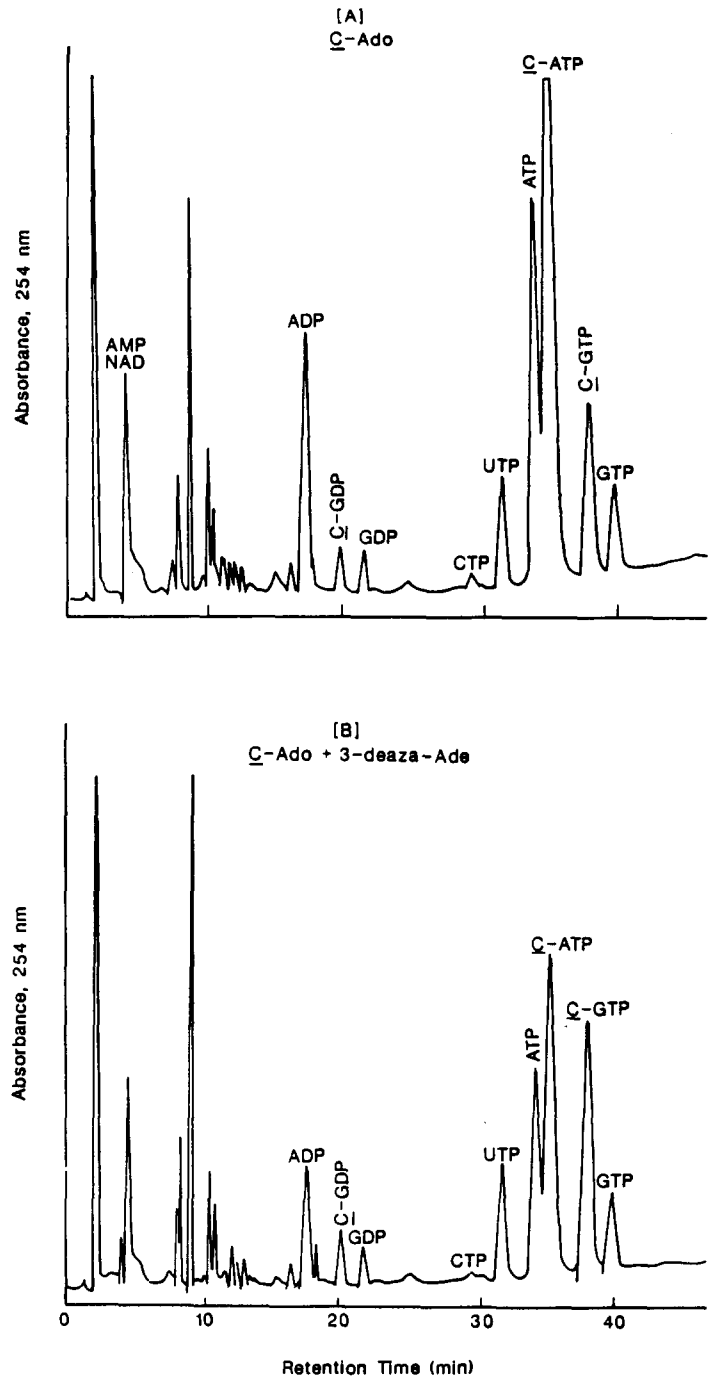

Fig. 7. Modification of the metabolism of *C*-Ado by 3-deaza-Ade. To L1210/0 cells in suspension culture (approximately 5×10^5 cells/ml), 3-deaza-Ade was added to a final concentration of 100 μM; 0.5 hr thereafter *C*-Ado was added at a concentration of 25 μM. Two hours after the addition of *C*-Ado the cells were harvested and extracted, and the nucleotide contents were analyzed by HPLC (see text).

Ado had the same effects on the utilization of labeled hypoxanthine and adenine: with each labeled precursor it produced an increase of ^{14}C in IMP and a decrease of ^{14}C in ATP (Fig. 3). In contrast, alanosine, a known inhibitor of adenylosuccinate synthase [16–18], inhibited the conversion of hypoxanthine to adenine nucleotides, as would be expected from its known mode of action, but had no marked effect on the metabolism of adenine (Fig. 4). The difference in the effects of 3-deaza-Ado and alanosine on the utilization of adenine would appear to eliminate the possibility that the effects of 3-deaza-Ado on nucleotide pools result from a block between IMP and AMP. One must therefore consider the alternative

possibility: stimulation of AMP-DA. None of the data is inconsistent with this mechanism. In addition, the experiment with *C*-Ado and 3-deaza-Ade (Fig. 7) provides strong indirect evidence for increased activity of AMP-DA. In this experiment the conversion of *C*-Ado to *C*-GTP was increased in the presence of 3-deaza-Ade and its conversion to *C*-ATP was decreased, as would be predicted if 3-deaza-Ade increased the activity of AMP-DA. Furthermore, a consideration of the literature on AMP-DA indicates a conceivable metabolic basis for this stimulation. AMP-DA is a regulatory enzyme and its activity is controlled by the size of the ATP and GTP pools [26]. ATP has been shown to stimulate many-fold the activity of AMP-DA from various sources [26], and we have found that the enzyme from L1210 cells is similarly stimulated.* We are not aware of any reports that an adenosine analog stimulates AMP-DA activity in intact cells. However, certain ATP analogs, namely iso-ATP (the isomer of ATP in which the ribosyl linkage is to N-3) and certain phosphonate analogs, have been shown to activate AMP-DA *in vitro* [27–29]. It would therefore appear as the most likely explanation of the observed effects that nucleotides derived from 3-deaza-Ado or 3-deaza-*C*-Ado simulate the action of ATP in stimulating AMP-DA. Unfortunately, this possibility has not been examined directly because we were unable to prepare 3-deaza-ATP in sufficient quantity. It is to be noted that this explanation would require that 3-deaza-ATP, which was present in cells in very small concentrations, be more potent than ATP as a stimulator, and that 3-deaza-*C*-ATP, which (if present) was below detectable levels, must be more potent still. Thus, although the data clearly point to a stimulation of AMP-DA in 3-deazapurine-treated cells, the mechanism for such stimulation is unproven.

The significance of these findings is 2-fold: (a) they illustrate a hitherto undescribed metabolic effect of 3-deaza-Ade, 3-deaza-Ado and 3-deaza-*C*-Ado and (b) they represent the first indication that a nucleoside analog may alter the activity of AMP-DA. The former would appear the less important. It is doubtful, for instance, if the effects on nucleotide pools contribute much to the overall biological effects of the 3-deazapurine nucleosides, because, at lower concentrations than those required to alter nucleotide pools, these agents inhibit AdoHcy hydrolase. The importance of the latter derives from the indication that it may be possible to modulate, by the use of nucleoside analogs, the activity of AMP-DA in intact cells. AMP-DA is an important enzyme of nucleotide metabolism and agents that specifically modulate its activity would be of obvious interest. The 3-deazapurines, because of lack of specificity, would not be expected to be useful for this purpose. But these results point to the possibility that other nucleosides might be designed that would be more effective and specific as stimulators of AMP-DA and that stimulation of AMP-DA may be a factor in the activity of other known adenosine analogs.

The finding of phosphates of 3-deaza-Ado and 3-deaza-*C*-Ado in L1210 cells is a point of some interest. In the early work on 3-deaza-Ado no evidence was found for nucleotide formation [21], but Zimmerman and co-workers [4, 30] have later reported 3-deaza-Ado to be slowly phosphorylated in lymphocytes. The amount of 3-deaza-AMP + 3-deaza-ATP formed in L1210 cells in our experiments is small (approx. 125 nmol/10^9 cells when the concentration of 3-deaza-Ado was 100 μM). The demonstrated conversion of 3-deaza-*C*-Ado to 3-deaza-*C*-AMP is the first evidence that this analog is phosphorylated. Although we were unable to demonstrate the presence of 3-deaza-*C*-ATP in cells grown in the presence of 3-deaza-*C*-Ado, it is likely that it was present because both *C*-Ado [12] and 3-deaza-Ado (Fig. 5) are converted to triphosphates. The methods used did not involve the use of radioactive nucleoside analogs, and 3-deaza-*C*-ATP might have been present but undetected because of small pool size and co-elution with natural triphosphates, as was in fact the case in our initial attempts to find 3-deaza-ATP. The enzyme responsible for the phosphorylation of 3-deaza-Ado and 3-deaza-*C*-Ado is also a question of interest. The fact that 3-deaza-ATP is formed in L1210/MeMPR cells in about the same amounts as in L1210/0 cells indicates that adenosine kinase is not solely responsible for its phosphorylation; this conclusion is in accord with the observation of Miller *et al.* [31] that 3-deaza-Ado is a very poor substrate for adenosine kinase. Formation of monophosphates from 3-deaza-Ado and 3-deaza-*C*-Ado could be demonstrated in cell-free preparations of L1210 cells supplemented with ATP, but this fact, while it confirms the result with intact cells, does not elucidate the mechanism of phosphorylation. Phosphorylation conceivably could be catalyzed by a kinase other than adenosine kinase, by a nucleoside phosphotransferase [32], or by the reverse action of a nucleotidase, as has been shown recently for the phosphorylation of both natural and unnatural nucleosides [33–35].

The moderate toxicity of 3-deaza-Ade to HEp-2 cells was surprising in view of its low toxicity to the L1210 cell lines (Table 1) and to D98 and L-cells [36]. Although this agent was first synthesized in 1949 [37], there is no information on its biological activity other than an early report of antibacterial activity [38] and a recent observation [36] of some activity as an anti-inflammatory agent.

* P. W. Allan and L. L. Bennett, Jr., unpublished observations.

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